IN THE UNITED STATES PATENT AND TRADEMARK OFFICE #10

In re application of:
Vic C. Knauf *et al.*
Appl. No.: 09/782,130
Filed: February 12, 2001
For: **Methods and Compositions for Regulated Transcription and Expression of Heterologous Genes**

Art Unit: To Be Assigned
Examiner: To Be Assigned
Atty. Docket: 16518.052

Preliminary Amendment

Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination on the merits, Applicants hereby request entry of the following amendments in the above-captioned application:

In the Specification:

Please **delete** the paragraph beginning on page 1, line 6, following the title of the application, and **replace** it with the following paragraph.

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Patent Application Serial No. 09/232,861, filed January 15, 1999, now U.S. Patent No. 6,281,410, which is a continuation of U.S. Patent Application Serial No. 08/812,665, filed March 7, 1997, now U.S. Patent No. 5,981,839, which is a continuation of U.S. Patent Application Serial No. 08/484,941, filed June 7, 1995, now U.S. Patent No. 5,750,385, which is a continuation of U.S. Patent Application Serial No. 08/105,852, filed August 10, 1993, now U.S. Patent No. 5,753,475. U.S. Patent Application Serial No. 08/105,852 is a continuation in part of U.S. Patent Application Serial No. 07/526,123, filed May 21, 1990, pending, which is a continuation of U.S. Patent Application Serial No. 07/267,685, filed November 2, 1988, now abandoned, which is a continuation of U.S. Patent Application Serial No. 06/692,605, filed January 17 1995, now abandoned. U.S.

Patent Application Serial No. 08/105,852 is also a continuation in part of U.S. Patent Application Serial No. 07/582,241, filed September 14, 1990, now abandoned, which is a continuation of U.S. Patent Application Serial No. 07/188,361, filed April 29, 1988, now abandoned, which is a continuation in part of U.S. Patent Application Serial No. 07/168,190, filed March 15, 1988, now abandoned, which is a continuation in part of U.S. Patent Application Serial No. 07/054,369, filed May 26, 1987, now U.S. Patent No. 4,943,674. U.S. Patent Application Serial No. 08/105,852 is also a continuation in part of U.S. Patent Application Serial No. 07/742,834, filed August 8, 1991, now U.S. Patent No. 5,420,034, which is a continuation in part of U.S. Patent Application Serial No. 07/550,804, filed July 9, 1990, now abandoned, which is a continuation in part of U.S. Patent Application Serial No. 07/147,781, filed January 25, 1988, now abandoned, which is a continuation in part of U.S. Patent Application Serial No. 07/078,538, filed July 28, 1987, now abandoned, which is a continuation in part of U.S. Patent Application Serial No. 06/891,529, filed July 31, 1986, now abandoned.

Please **add** the following paragraph on page 1 after the paragraph entitled "Cross reference to Related Applications":

INCORPORATION OF SEQUENCE LISTING

A paper copy of the Sequence Listing and a computer readable form of the sequence listing on diskette, containing the file named seqlst.txt, which is 48,475 bytes in size (measured in MS-DOS), and which was created on October 21, 2002, are herein incorporated by reference.

Please **delete** the paragraphs spanning page 7, line 2 through page 8, line 11, following the Brief Description of the Drawings, and **replace** them with the following paragraphs.

Figure 1 is a partial sequence (SEQ ID NO: 1) of the promoter region of the λBnNa napin gene. The start (ATG) codon of the open reading frame is underlined.

Figure 2 is a restriction map of cloned λCGN1-2 (SEQ ID NO: 2) showing the entire coding region sequence (SEQ ID NO: 3) as well as extensive 5' upstream and 3' downstream sequences.

Figure 3 is a partial nucleotide sequence (SEQ ID NO: 4) of genomic ACP clone Bcg4-4. The coding region (SEQ ID NO: 5) is indicated by the three-letter amino acid codes. Breaks in the coding region sequence represent introns. The underlined nucleotide at position 310 is ambiguous without further sequence analysis for confirmation.

Figure 4 is the complete nucleotide sequence (SEQ ID NO: 6) of *B. campestris* cDNA EA9. The longest open reading frame (SEQ ID NO: 7) is designated by the three letter amino acid code. PloyA tails are evident at the end of the sequence and a potential polyadenylation signal is underlined.

Figure 5 shows the nucleotide sequence of the cDNA clones PCGN1299 (2A11) (SEQ ID NO: 8) and PCGN1298 (3H11) (SEQ ID NO: 9). The amino acid sequence of the polypeptide (SEQ ID NO: 10) encoded by the open reading frame is also indicated.

Figure 6 is a comparison of 2A11 to pea storage proteins and other abundant storage proteins:

(a) 2A11 (residues 33-46) (SEQ ID NO: 11) is compared to PA1b and the reactive site sequences of some protease inhibitors, Pa1b (residues 6-23) (SEQ ID NO: 12), chick pea inhibitor (residues 11-23) (SEQ ID NO: 13), lime bean inhibitor (residues 23-35) (SEQ ID NO: 14), human α1-antitrypsin (SEQ ID NO: 15) reactive site peptide. The arrow indicates the reactive site.

(b) is a comparison of the amino terminal sequence of 2A11 (SEQ ID NO: 16) with the amino termini of a range of seed proteins. The data have been modified or deletions introduced to maximize homology; conserved residues are shown boxed. The sequences are from the following sources: PA1b (SEQ ID NO: 17); barley chloroform/methanol-soluable protein d (SEQ ID NO: 18); wheat albumin (SEQ ID NO: 20); wheat α-amylase inhibitor 0.28 (SEQ ID NO: 19); millet bi-functional inhibitor (SEQ ID NO: 21); castor bean 2S small subunit (SEQ ID NO: 22); and napin small subunit (SEQ ID NO: 23).

Figure 7 shows the complete sequence of the 2A11 genomic DNA (SEQ ID NO: 24) and protein (SEQ ID NO: 25) cloned into PCGN1273 from the XhoI site (position 1 at the 5' end) to the EcoRI site (position 4654).

Figure 8 shows the nucleotide sequence (SEQ ID NO: 26) of a polygalacturonase (PG) genomic clone.

Please **delete** the paragraph beginning at page 35, line 3, following Example 2, "Construction of a Napin Promoter", and **replace** it with the following paragraph.

There are 298 nucleotides upstream of the ATG start codon of the napin gene on the pgN1 clone, a 3.3 kb *Eco*RI fragment of B. napus genomic DNA containing a napin gene cloned into pUC8 (available from Marti Crouch, University of Indiana). pgN1 DNA was digested with *Eco*RI and *Sst*I and ligated to *Eco*RI/*Sst*I digested pCGN706. (pCGN706 is an *Xho*I/*Pst*I fragment containing 3' and polyadenylation sequences of another napin cDNA clone pN2 (Crouch *et al.*, 1983 supra) cloned in pCGN566 at the *Sal*I and *Pst*I sites.) The resulting clone pCGN707 was digested with *Sal*I and treated with the enzyme *Bal*31 to remove some of the coding region of the napin gene. The resulting resected DNA was digested with *Sma*I after the *Bal*31 treatment and religated. One of the clones, pCGN713, selected by size, was subcloned by *Eco*RI and *Bam*HI digestion into both *Eco*RI-*Bam*HI digested pEMBL18 (Dente *et al.*, *Nucleic Acids Res.* (1983) *11*:1645-1655) and pUC118 to give E418 and E4118 respectively. The extent of *Bal*31 digestion was confirmed by Sanger dideoxy sequencing of E418 template. The *Bal*31 deletion of the promoter region extended only to 57 nucleotides downstream of the start codon, thus containing the 5' end of the napin coding sequence and about 300 bp of the 5' non-coding region. E4118 was tailored to delete all of the coding region of napin including the ATG start codon by in vitro mutagenesis by the method of Zoller and Smith (*Nucleic Acids Res.* (1982) *10*:6487-6500) using an oligonucleotide primer 5'-GATGTTTTGTATGTGGGCCCCTAGGAGATC-3' (SEQ ID NO: 27). Screening for the appropriate mutant was done by two transformations into *E. coli* strain JM83 (Messing J., In: Recombinant DNA Technical Bulletin, NIH Publication No. 79-99, *2* No. 2, 1979, pp 43-48) and *Sma*I digestion of putative transformants. The resulting

napin promoter clone is pCGN778 and contains 298 nucleotides from the *Eco*RI site of pgN1 to the A nucleotide just before the ATG start codon of napin. The promoter region was subcloned into a chloramphenicol resistant background by digestion with *Eco*RI and *Bam*HI and ligation to *Eco*RI-*Bam*HI digested pCGN565 to give pCGN779c.

Please **delete** the paragraph beginning at page 37, line 26, following "Identification of a Spinach ACP-I cDNA", and **replace** it with the following paragraph.

A total of approximately 8000 cDNA clones were screened by performing Southern blots (Southern, *J. Mol. Biol.* (1975) *98*:503) and dot blot (described below) hybridizations with clone analysis DNA from 40 pools representing 200 cDNA clones each (see below). A 5' end-labeled synthetic oligonucleotide (ACPP4) that is at least 66% homologous with a 16 amino acid region of spinach ACP-I (5'-GATGTCTTGAGCCTTGTCCTCATCCACATTGA TACCAAACTCCTCCTC-3') (SEQ ID NO: 28) is the complement to a DNA sequence that could encode the 16 amino acid peptide glu-glu-glu-phe-gly-ile-asn-val-asp-glu-asp-lys-ala-gln-asp-ile (SEQ ID NO: 29), residues 49-64 of spinach ACP-I (Kuo and Ohlrogge, Arch. *Biochem. Biophys.* (1984) *234*:290-296) and ease used for an ACP probe.

Please **delete** the paragraphs spanning page 52, line 23 through page 53, line 19 and **replace** them with the following paragraphs.

One of the clones named lambda CGN1-2 was restriction mapped and the napin gene was localized to overlapping 2.7 kb *Xho*I and 2.1 kb *Sal*I restriction fragments. The two fragments were subcloned from lambda CGN1-2 DNA into pCGN789 (a pUC based vector the same as pUC119 with the normal polylinker replaced by the synthetic linker—5' GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3' (SEQ ID NO: 30) (which represents the polylinker *Eco*RI, *Sal*I, *Bgl*II, *Pst*I, *Xho*I, *Bam*HI, *Hind*III). The identity of the subclones as napin was confirmed by sequencing. The entire coding region sequence as well as extensive 5' upstream and 3' downstream sequences were determined (FIG. 2). The lambda CGN1-2 napin gene is that encoding the mRNA corresponding to the BE5 cDNA as determined by the exact match of their nucleotide sequence.

An expression cassette was constructed from the 5'-end and the 3'-end of the lambda CGN1-2 napin gene as follows in an analogous manner to the construction of pCGN944. The majority of the napin coding region of pCGN940 was deleted by digestion with *Sal*I and religation to form pCGN1800. Single-stranded DNA from pCGN1800 was used in an in vitro mutagenesis reaction (Adelman *et al.*, DNA (1983) *2*:183-193) using the synthetic oligonucleotide 5' GCTTGTTCGCCATGGATATCTTCTGTATGTTC 3' (SEQ ID NO: 31). This oligonucleotide inserted an *Eco*RV and an *Nco*l restriction site at the junction of the promoter region and the ATG start codon of the napin gene. An appropriate mutant was identified by hybridization to the oligonucleotide used for the mutagenesis and sequence analysis and named pCGN1801.

Please **delete** the paragraph beginning at page 55, line 27, following Example 4, "Isolation of Other Seed Specific Promoters", and **replace** it with the following paragraph.

Other seed-specific promoters may be isolated from genes encoding proteins involved in seed triacylglycerol synthesis, such as acyl carrier protein from *Brassica* seeds. Immature seeds were collected from *Brassica campestris* cv. "R-500," a self-compatible variety of turnip rape. Whole seeds were collected at stages corresponding approximately to 14 to 28 days after flowering. RNA isolation and preparation of a cDNA bank was as described above for the isolation of a spinach ACP cDNA clone except the vector used was pCGN565. To probe the cDNA bank, the oligonucleotide (5')-ACTTTCTCAACTGTCTCTGGTTTAGC AGC-(3') (SEQ ID NO: 32) was synthesized using an Applied Biosystems DNA Synthesizer, model 380A, according to manufacturer's recommendations. This synthetic DNA molecule will hybridize at low stringencies to DNA or RNA sequences coding for the amino acid sequence (ala-ala-lys-pro-glu-thr-val-glulys-val) (SEQ ID NO: 33). This amino acid sequence has been reported for ACP isolated from seeds of *Brassica napus* (Slabas *et al.*, 7th International Symposium of the Structure and Function of Plant Lipids, University of California, Davis, Calif., 1986); ACP from *B. campestris* seed is highly homologous. Approximately 2200 different cDNA clones were analyzed using a colony hybridization technique (Taub and Thompson, *Anal. Biochem.* (1982) *126*:222-230) and hybridization

conditions corresponding to Wood *et al.* (*Proc. Natl. Acad. Sci.* (1985) *82*:1585-1588). DNA sequence analysis of two cDNA clones showing obvious hybridization to the oligonucleotide probe indicated that one, designated pCGN1Bcs, indeed coded for an ACP-precursor protein by the considerable homology of the encoded amino acid sequence with ACP proteins described from *Brassica napus* (Slabas *et al.*, 1980 *supra*). Similarly to Example 3, the ACP cDNA clone, pCGN1BCS, was used to isolate ACP genomic clones containing the regulatory information for expression of ACP during triacylglyceride synthesis in the seeds. DNA was isolated from *B. campestris* cv. R500 young leaves by the procedure of Scofield and Crouch (*J. Biol.Chem.* (1987) *262*:12202-12208). A Sau3A partial genomic library of the *B. campestris* DNA was made in the lambda vector Embl 3 (Stratagene, San Diego, Calif.) using established protocols (Maniatis *et al.*, (1982) *supra*) and manufacturer's instructions. The titer of the library was -1.0x10^8 phage/ml. Six hundred thousand recombinant bacteriophage were plated and screened as described in Example 3 with the exception that the *E. coli* host cells used were strain P2392 (Stratagene, San Diego, Calif.). Filters were prehybridized and hybridized at 42°C in 25 ml each of hybridization buffer containing 50% formamide, 10x Denhardt's, 5xSSC, 5 Mm EDTA, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA (reagents described in Maniatis *et al.*, (1982) *supra*). The probe used in these hybridizations was 0.2 µg of a nick-translated 530 base pair *Bgl*II-*Dra*I fragment of pCGN1Bcs, the *B. campestris* ACP cDNA clone described above. Six plaques were hybridized strongly on duplicate filters after washing the filters at 55°C in 0.1xSSC/0.2% SDS, and were plaque-purified as described (Maniatis *et al.*, (1982) *supra*).

Please **delete** the paragraph beginning at page 58, line 17 and **replace** it with the following paragraph.

An expression cassette can be constructed from the 5' upstream sequences and 3' downstream sequences of Bcg4-4 as follows. The pCGN1941 *Xho*I subclone is used for the 5' regulatory region. This clone contains the *Xho*I insert in the opposite orientation of the lacZ gene. The 3' regulatory region is altered to allow cloning as a *Pst*I-*Bgl*II fragment into pCGN565 by oligonucleotide site-directed mutagenesis. Single-stranded DNA is made from

pCGN1940 and altered by mutagenesis as described (Adelman et al., supra) with the synthetic oligonucleotide 5' CTTAAGAAGTAACCCGGGCTGCAGTTTTAGTATTA AGAG 3' (SEQ ID NO: 34). This oligonucleotide provides *Sma*I and *Pst*I restriction sites just after the TAA stop codon of the pCGN1Bcs cDNA. The PstI-BglII 3' fragment is then cloned into the *Pst*I and *Bam*HI sites (the *Bam*HI restriction site is destroyed in this process) of pCGNS565. The resulting clone is digested with *Pst*I and *Sma*I, and the fragment inserted into the corresponding sites in pCGN1941 (described above) in the same orientation as the 5' region. The resulting clone comprises the ACP expression cassette with *Pst*I, *Eco*RI, and *Eco*RV sites available between the 5' and 3' regulatory regions for the cloning of genes to be expressed under the regulation of these ACP gene regions.

Please **delete** the paragraph beginning at page 77, line 11, after "Construction of Plasmid pCGN1241", and **replace** it with the following paragraph.

A more convenient version has the *Eco*RI of pCGN1240 excised and inserted into a Bluescript vector called pCGN1239 which has an altered polylinker region such that the entire cassette can be excised as a *Sac*I-*Kpn*I fragment. The altered Bluescript vector, pCGN1239, was constructed by modifying the BlueScript polybinder from the *Sac*I site to the KpnI site including a synthetic polylinker with the following sequence: AGCTCGGTACCG AATTCGAGCTCGGTAC (SEQ ID NO: 35) to create a polylinker with the following sites: *Sac*I-*Kpn*I-*Eco*RI *Sac*I-*Kpn*I. The *Eco*RI insert of pCGN1240 was inserted into pCGN1239 to make pCGN1241 (see FIG. 9).

Remarks

The specification has been amended to update the priority data and to make explicit reference to the Sequence Listing provided in computer readable form in the present application. The specification has also been amended to make reference to the SEQ ID NOs provided in the Sequence Listing where applicable. No new matter enters by these amendments.

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is now in condition for allowance, and notice of such is respectfully requested. The Examiner is encouraged to contact the undersigned should any additional information be necessary for allowance.

In the event that extensions of time beyond those petitioned for herewith are necessary to prevent abandonment of this patent application, then such extensions of time are hereby petitioned. Applicants do not believe that any fees in addition to the petition fee are required at this time. However, if any fees under 37 C.F.R. 1.16 or 1.17 are required in the present application, including any fees for extensions of time, then the Commissioner is hereby authorized to charge such fees to Deposit Account No. 50-2387, referencing docket number 16518.052.

Respectfully submitted,

Holly Logue Prutz (Reg. No. 47,755)
June E. Cohan (Reg. No. 43,741)

Date: October 22, 2002

ARNOLD & PORTER
555 Twelfth Street, N.W.
Washington, D.C. 20004-1206
(202) 942-5000 telephone
(202) 942-5999 facsimile

Marked-up Version Showing Changes Made

At page 1, line 6, following the title of the application:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of **[U.S.S.N.] U.S. Patent Application Serial No.** 09/232,861, filed January 15, 1999, **now U.S. Patent No. 6,281,410,** which is a continuation of **[U.S.S.N.] U.S. Patent Application Serial No.** 08/812,665, filed March 7, 1997, now U.S. Patent No. 5,981,839, which is a continuation of **[U.S.S.N.] U.S. Patent Application Serial No.** 08/484,941, filed June 7, 1995, **now U.S. Patent No. 5,750,385,** which is a continuation of **[U.S.S.N.] U.S. Patent Application Serial No.** 08/105,852, filed **[8/10/93] August 10, 1993, [pending] now U.S. Patent No. 5,753,475. [;] [U.S.S.N.] U.S. Patent Application Serial No.** 08/105,852 is a continuation in part of **U.S. Patent Application Serial No.** 07/526,123, filed **[5/21/90] May 21, 1990**, pending, which is a continuation of **U.S. Patent Application Serial No.** 07/267,685, filed **[11/2/88] November 2, 1988, now** abandoned, which is a continuation of **U.S. Patent Application Serial No.** 06/692,605, filed **[1/17/85,] January 17, 1995, now** abandoned**[;].** **[U.S.S.N.] U.S. Patent Application Serial No.** 08/105,852**[,]** is also a continuation in part of **U.S. Patent Application Serial No.** 07/582,241, filed **[9/14/90,] September 14, 1990, now** abandoned, which is a continuation of **U.S. Patent Application Serial No**. 07/188,361, filed **[4/29/88,] April 29, 1988, now** abandoned, which is a continuation in part of **U.S. Patent Application Serial No.** 07/168,190, filed **[3/15/88,] March 15, 1988, now** abandoned, which is a continuation in part of **U.S. Patent Application Serial No.** 07/054,369, filed **[5/26/87, which issued on 7/24/90 as patent number 4,943,674;] May 26, 1987, now U.S. Patent No. 4,943,674. [U.S.S.N.] U.S. Patent Application Serial No.** 08/105,852 is also a continuation in part of **[U.S.S.N.] U.S. Patent Application Serial No.** 07/742,834, **filed** August 8, 1991, **[which issued as U.S. Patent No. 5,420,034 issued on 5/30/95,] now U.S. Patent No. 5,420,034,** which is a continuation in part of **U.S. Patent Application Serial No.** 07/550,804, filed **[7/9/90,] July 9, 1990, now** abandoned, which is a continuation in part of **U.S. Patent Application Serial No.**

07/147,781, filed **[1/25/88,] January 25, 1988, now** abandoned, which is a continuation in part of **U.S. Patent Application Serial No.** 07/078,538, filed **[7/28/87,] July 28, 1987, now** abandoned, which is a continuation in part of **U.S. Patent Application Serial No.** 06/891,529, filed **[7/31/86, which is abandoned.] July 31, 1986, now abandoned.**

At page 7, line 2 through page 8, line 11, following the Brief Description of the Drawings:

Figure 1 is a partial sequence **(SEQ ID NO: 1)** of the promoter region of the λBnNa napin gene. The start (ATG) **codon** of the open reading frame is underlined.

Figure 2 is a restriction map of cloned λCGN1-2 **(SEQ ID NO: 2)** showing the entire coding region sequence **(SEQ ID NO: 3)** as well as extensive 5' upstream and 3' downstream sequences.

Figure 3 is a partial nucleotide sequence **(SEQ ID NO: 4)** of genomic ACP clone Bcg4-4. The coding region **(SEQ ID NO: 5)** is indicated by the three-letter amino acid codes. Breaks in the coding region sequence represent introns. The underlined nucleotide at position 310 is ambiguous without further sequence analysis for confirmation.

Figure 4 is the complete nucleotide sequence **(SEQ ID NO: 6)** of *B. campestris* cDNA EA9. The longest open reading frame **(SEQ ID NO: 7)** is designated by the three letter amino acid code. PloyA tails are evident at the end of the sequence and a potential polyadenylation signal is underlined.

Figure 5 shows the nucleotide sequence of the cDNA clones PCGN1299 (2A11) **(SEQ ID NO: 8)** and PCGN1298 (3H11) **(SEQ ID NO: 9)**. The amino acid sequence of the polypeptide **(SEQ ID NO: 10)** encoded by the open reading frame is also indicated.

Figure 6 is a comparison of 2A11 to pea storage proteins and other abundant storage proteins:

(a) 2A11 (residues 33-46) **(SEQ ID NO: 11)** is compared to PA1b and the reactive site sequences of some protease inhibitors, Pa1b (residues 6-23) **(SEQ ID NO: 12)**, chick pea inhibitor (residues 11-23) **(SEQ ID NO: 13)**, lime bean inhibitor (residues 23-35)

(SEQ ID NO: 14), human α1-antitrypsin **(SEQ ID NO: 15)** reactive site peptide. The arrow indicates the reactive site.

(b) is a comparison of the amino terminal sequence of 2A11 **(SEQ ID NO: 16)** with the amino termini of a range of seed proteins. The data have been modified or deletions introduced to maximize homology; conserved residues are shown boxed. The sequences are from the following sources: PA1b **(SEQ ID NO: 17)**; barley chloroform/methanol-soluable protein d **(SEQ ID NO: 18)**; wheat albumin **(SEQ ID NO: 20)**; wheat α-amylase inhibitor 0.28 **(SEQ ID NO: 19)**; millet bi-functional inhibitor **(SEQ ID NO: 21)**; castor bean 2S small subunit **(SEQ ID NO: 22)**; and napin small subunit **(SEQ ID NO: 23)**.

Figure 7 shows the complete sequence of the 2A11 genomic DNA **(SEQ ID NO: 24) and protein (SEQ ID NO: 25)** cloned into PCGN1273 from the XhoI site (position 1 at the 5' end) to the EcoRI site (position 4654).

Figure 8 shows the nucleotide sequence **(SEQ ID NO: 26)** of a polygalacturonase (PG) genomic clone.

At page 35, line 3, following Example 2, "Construction of a Napin Promoter":

There are 298 nucleotides upstream of the ATG start codon of the napin gene on the pgN1 clone, a 3.3 kb *Eco*RI fragment of B. napus genomic DNA containing a napin gene cloned into pUC8 (available from Marti Crouch, University of Indiana). pgN1 DNA was digested with *Eco*RI and *Sst*I and ligated to *Eco*RI/*Sst*I digested pCGN706. (pCGN706 is an *Xho*I/*Pst*I fragment containing 3' and polyadenylation sequences of another napin cDNA clone pN2 (Crouch *et al.*, 1983 supra) cloned in pCGN566 at the *Sal*I and *Pst*I sites.) The resulting clone pCGN707 was digested with *Sal*I and treated with the enzyme *Bal*31 to remove some of the coding region of the napin gene. The resulting resected DNA was digested with *Sma*I after the *Bal*31 treatment and religated. One of the clones, pCGN713, selected by size, was subcloned by *Eco*RI and *Bam*HI digestion into both *Eco*RI-*Bam*HI digested pEMBL18 (Dente *et al.*, *Nucleic Acids Res.* (1983) *11*:1645-1655) and pUC118 to give E418 and E4118 respectively. The extent of *Bal*31 digestion was confirmed by Sanger dideoxy sequencing of E418 template. The *Bal*31 deletion of the promoter region extended

only to 57 nucleotides downstream of the start codon, thus containing the 5' end of the napin coding sequence and about 300 bp of the 5' non-coding region. E4118 was tailored to delete all of the coding region of napin including the ATG start codon by in vitro mutagenesis by the method of Zoller and Smith (*Nucleic Acids Res.* (1982) *10*:6487-6500) using an oligonucleotide primer 5'-GATGTTTTGTATGTGGGCCCCTAGGAGATC-3' **(SEQ ID NO: 27)**. Screening for the appropriate mutant was done by two transformations into *E. coli* strain JM83 (Messing J., In: Recombinant DNA Technical Bulletin, NIH Publication No. 79-99, *2* No. 2, 1979, pp 43-48) and *Sma*I digestion of putative transformants. The resulting napin promoter clone is pCGN778 and contains 298 nucleotides from the *Eco*RI site of pgN1 to the A nucleotide just before the ATG start codon of napin. The promoter region was subcloned into a chloramphenicol resistant background by digestion with *Eco*RI and *Bam*HI and ligation to *Eco*RI-*Bam*HI digested pCGN565 to give pCGN779c.

At page 37, line 26, following "Identification of a Spinach ACP-I cDNA":

A total of approximately 8000 cDNA clones were screened by performing Southern blots (Southern, *J. Mol. Biol.* (1975) *98*:503) and dot blot (described below) hybridizations with clone analysis DNA from 40 pools representing 200 cDNA clones each (see below). A 5' end-labeled synthetic oligonucleotide (ACPP4) that is at least 66% homologous with a 16 amino acid region of spinach ACP-I (5'-GATGTCTTGAGCCTTGTCCTCATCCACATTGA TACCAAACTCCTCCTC-3') **(SEQ ID NO: 28)** is the complement to a DNA sequence that could encode the 16 amino acid peptide glu-glu-glu-phe-gly-ile-asn-val-asp-glu-asp-lys-ala-gln-asp-ile **(SEQ ID NO: 29)**, residues 49-64 of spinach ACP-I (Kuo and Ohlrogge, Arch. *Biochem. Biophys.* (1984) *234*:290-296) and ease used for an ACP probe.

At page 52, line 23 through page 53, line 19:

One of the clones named lambda CGN1-2 was restriction mapped and the napin gene was localized to overlapping 2.7 kb *Xho*I and 2.1 kb *Sal*I restriction fragments. The two fragments were subcloned from lambda CGN1-2 DNA into pCGN789 (a pUC based vector the same as pUC119 with the normal polylinker replaced by the synthetic linker—5'

GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3' **(SEQ ID NO: 30)** (which represents the polylinker *Eco*RI, *Sal*I, *Bgl*II, *Pst*I, *Xho*I, *Bam*HI, *Hind*III). The identity of the subclones as napin was confirmed by sequencing. The entire coding region sequence as well as extensive 5' upstream and 3' downstream sequences were determined (FIG. 2). The lambda CGN1-2 napin gene is that encoding the mRNA corresponding to the BE5 cDNA as determined by the exact match of their nucleotide sequence.

An expression cassette was constructed from the 5'-end and the 3'-end of the lambda CGN1-2 napin gene as follows in an analogous manner to the construction of pCGN944. The majority of the napin coding region of pCGN940 was deleted by digestion with *Sal*I and religation to form pCGN1800. Single-stranded DNA from pCGN1800 was used in an in vitro mutagenesis reaction (Adelman *et al.*, DNA (1983) *2*:183-193) using the synthetic oligonucleotide 5' GCTTGTTCGCCATGGATATCTTCTGTATGTTC 3' **(SEQ ID NO: 31)**. This oligonucleotide inserted an *Eco*RV and an *Nco*I restriction site at the junction of the promoter region and the ATG start codon of the napin gene. An appropriate mutant was identified by hybridization to the oligonucleotide used for the mutagenesis and sequence analysis and named pCGN1801.

At page 55, line 27, following Example 4, "Isolation of Other Seed Specific Promoters":

Other seed-specific promoters may be isolated from genes encoding proteins involved in seed triacylglycerol synthesis, such as acyl carrier protein from *Brassica* seeds. Immature seeds were collected from *Brassica campestris* cv. "R-500," a self-compatible variety of turnip rape. Whole seeds were collected at stages corresponding approximately to 14 to 28 days after flowering. RNA isolation and preparation of a cDNA bank was as described above for the isolation of a spinach ACP cDNA clone except the vector used was pCGN565. To probe the cDNA bank, the oligonucleotide (5')-ACTTTCTCAACTGTCTCTGGTTTAGC AGC-(3') **(SEQ ID NO: 32)** was synthesized using an Applied Biosystems DNA Synthesizer, model 380A, according to manufacturer's recommendations. This synthetic DNA molecule will hybridize at low stringencies to DNA or RNA sequences coding for the

amino acid sequence (ala-ala-lys-pro-glu-thr-val-glulys-val) **(SEQ ID NO: 33)**. This amino acid sequence has been reported for ACP isolated from seeds of *Brassica napus* (Slabas *et al.*, 7th International Symposium of the Structure and Function of Plant Lipids, University of California, Davis, Calif., 1986); ACP from *B. campestris* seed is highly homologous. Approximately 2200 different cDNA clones were analyzed using a colony hybridization technique (Taub and Thompson, *Anal. Biochem.* (1982) *126*:222-230) and hybridization conditions corresponding to Wood *et al.* (*Proc. Natl. Acad. Sci.* (1985) *82*:1585-1588). DNA sequence analysis of two cDNA clones showing obvious hybridization to the oligonucleotide probe indicated that one, designated pCGN1Bcs, indeed coded for an ACP-precursor protein by the considerable homology of the encoded amino acid sequence with ACP proteins described from *Brassica napus* (Slabas *et al.*, 1980 *supra*). Similarly to Example 3, the ACP cDNA clone, pCGN1BCS, was used to isolate ACP genomic clones containing the regulatory information for expression of ACP during triacylglyceride synthesis in the seeds. DNA was isolated from *B. campestris* cv. R500 young leaves by the procedure of Scofield and Crouch (*J. Biol.Chem.* (1987) *262*:12202-12208). A Sau3A partial genomic library of the *B. campestris* DNA was made in the lambda vector Embl 3 (Stratagene, San Diego, Calif.) using established protocols (Maniatis *et al.*, (1982) *supra*) and manufacturer's instructions. The titer of the library was -1.0x10^8 phage/ml. Six hundred thousand recombinant bacteriophage were plated and screened as described in Example 3 with the exception that the *E. coli* host cells used were strain P2392 (Stratagene, San Diego, Calif.). Filters were prehybridized and hybridized at 42°C in 25 ml each of hybridization buffer containing 50% formamide, 10x Denhardt's, 5xSSC, 5 Mm EDTA, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA (reagents described in Maniatis *et al.*, (1982) *supra*). The probe used in these hybridizations was 0.2 µg of a nick-translated 530 base pair *Bgl*II-*Dra*I fragment of pCGN1Bcs, the *B. campestris* ACP cDNA clone described above. Six plaques were hybridized strongly on duplicate filters after washing the filters at 55°C in 0.1xSSC/0.2% SDS, and were plaque-purified as described (Maniatis *et al.*, (1982) *supra*).

At page 58, line 17:

An expression cassette can be constructed from the 5' upstream sequences and 3' downstream sequences of Bcg4-4 as follows. The pCGN1941 *Xho*I subclone is used for the 5' regulatory region. This clone contains the *Xho*I insert in the opposite orientation of the lacZ gene. The 3' regulatory region is altered to allow cloning as a *Pst*I-*Bgl*II fragment into pCGN565 by oligonucleotide site-directed mutagenesis. Single-stranded DNA is made from pCGN1940 and altered by mutagenesis as described (Adelman et al., supra) with the synthetic oligonucleotide 5' CTTAAGAAGTAACCCGGGCTGCAGTTTTAGTATTA AGAG 3' **(SEQ ID NO: 34)**. This oligonucleotide provides *Sma*I and *Pst*I restriction sites just after the TAA stop codon of the pCGN1Bcs cDNA. The PstI-BglII 3' fragment is then cloned into the *Pst*I and *Bam*HI sites (the *Bam*HI restriction site is destroyed in this process) of pCGNS565. The resulting clone is digested with *Pst*I and *Sma*I, and the fragment inserted into the corresponding sites in **[P]p**CGN1941 (described above) in the same orientation as the 5' region. The resulting clone comprises the ACP expression cassette with *Pst*I, *Eco*RI, and *Eco*RV sites available between the 5' and 3' regulatory regions for the cloning of genes to be expressed under the regulation of these ACP gene regions.

At page 77, line 11, after "Construction of Plasmid pCGN1241":

A more convenient version has the *Eco*RI of pCGN1240 excised and inserted into a Bluescript vector called pCGN1239 which has an altered polylinker region such that the entire cassette can be excised as a *Sac*I-*Kpn*I fragment. The altered Bluescript vector, pCGN1239, was constructed by modifying the BlueScript polybinder from the *Sac*I site to the KpnI site including a synthetic polylinker with the following sequence: AGCTCGGTACCG AATTCGAGCTCGGTAC **(SEQ ID NO: 35)** to create a polylinker with the following sites: *Sac*I-*Kpn*I-*Eco*RI *Sac*I-*Kpn*I. The *Eco*RI insert of pCGN1240 was inserted into pCGN1239 to make pCGN1241 (see FIG. 9).